Relationship of some coleoid cephalopods established by 3' end of the 16S rDNA and cytochrome oxidase III gene sequence comparison

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Abstract: Phylogenetic relationships for extant cephalopods have been based, so far, mainly on morphology and paleontology. Nucleotide sequence data are still rare. Sequence analyses from the 3' end of the 16S rDNA gene of cephalopods have shown that this portion of gene can provide valuable information on taxonomic relationships at the infrafamilial level. Another mitochondrial gene, cytochrome oxidase III, is investigated to analyze higher (*i. e.* ordinal) taxonomic levels. The results obtained by the two gene portions are compared, but the low number of species does not allow a definitive answer on interfamilial relationships. The low divergence between nucleotide sequences of two populations of *Loligo vulgaris* Lamarck, 1798, and of *L. reynaudii* Orbigny, 1845, suggests that the latter is not a clearly distinct species. The grouping of the three families of Sepioidea (Sepiidae, Spirulidae, and Sepiolidae) is not supported. Idiosepiidae groups with the oegopsid squid *Enoploteuthis* irregardless of the analysis (parsimony or distance).

The Decapoda are composed of two orders, Teuthoidea and Sepioidea. Relationships within these orders are not stabilized based on morphological characters. It is now possible with molecular characters to obtain a new type of information which can help to resolve some phylogenetic relationships. The 3' end of the mitochondrial- *l-r-*RNA (16S) was already investigated but with this portion of the molecule, extensive nucleotide variability leads to an unresolved phylogeny between the orders (Bonnaud *et al.*, 1994). Another mitochondrial gene, coding for cytochrome oxidase III (COIII), was chosen here to analyze the relationships between some species of decapods. The impact of mutations on protein function is very important, and accordingly, the structure of some genes coding for proteins should be less variable at the nucleotide level: this is the case for the genes coding for cytochrome oxidase subunits, COI, COII, and COIII. COIII gene analyses were thus thought to be suitable for solving phylogenetic relationships at hierarchical taxonomic levels higher than those resolved by the 16S gene. For this preliminary study, one species in each family or suborder was analyzed and the results obtained with the two gene portions compared.

MATERIAL AND METHODS

Details on the taxonomic position and origin of the eight species studied are presented in Table 1. DNA was extracted from frozen or alcohol-preserved tissues according to the protocol described in Bonnaud *et al.* (1994). A portion of 16S and a portion of COIII were amplified with universal primers: 984 and 986 for 16S and COIIIa and COIIIb according to the classification of Simon *et al.* (1991). These portions were cloned in pBS+ (Stratagène) and sequenced (ca. 500 pb each) with the dideoxy chain termination (Sanger *et al.*, 1977). The alignments were performed by eye, with the aid of secondary structure for 16S and of the reading coding frame for COIII. Phylogenetic trees were calculated by distance (Neighbor-Joining) method using the MUST package (Philippe, 1992) and parsimony method using PAUP 3.1 (Swofford, 1990). These two methods gave similar results and only the trees obtained with the distance method are described here. The robustness of internal branching was tested by bootstrapping.

Transversions (changes of pyrimidine to purine or *vice versa*) are known to be less abundant than transitions (changes of purine to purine or pyrimidine to pyrimidine) in some vertebrate taxa. When sequences are highly variable, transitions can introduce noise in the analyses. As a consequence, the use of transversions should lower the incidence of homoplasy between distant taxa. Analyses were performed using both all the substitutions and only the transversions. Attributing weight to the transversions instead of removing the transitions did not further change the results obtained.

Table 1. Geographical origin and systematic position of species studied.

SPECIES (ORIGIN)	SYSTEMATIC POSITION	
Sepia officinalis Linné, 1758 (Banyuls)	Sepiidae	SEPIOIDEA
Sepietta sp. (Banyuls)	Sepiolidae	
Spirula spirula (Linné, 1758) (New Caledonia)	Spirulidae	
Idiosepius pygmaeus Steenstrup, 1881 (Australia)	Idiosepiidae	
Enoploteuthis reticulata Rancurel, 1970 (Hawaii)	Enoploteuthidae (Oegopsid squid)	TEUTHOIDEA
Loligo vulgaris Lamarck, 1798 (Roscoff)	Loliginidae (Myopsid squids)	
L. vulgaris (Banyuls)		
L. reynaudii Orbigny, 1845 (South Africa)		
Octopus cyanea Gray, 1849 (New Caledonia)	Octopodidae	

RESULTS

Analysis of partial 16S gene

Analyses were performed with 131 informative sites out of 231 variable sites. In agreement with the results obtained previously (Bonnaud *et al.*, 1994) the tree issued from the analysis of *l-r*-RNA gene is unresolved (Fig. 1). No strong relationship can be established among taxa, except for the Loliginidae. This is correlated with the sequence identities: the sequences of the two *Loligo vulgaris* populations are identical and that of *L. reynaudii* differs by only 1.1%, a percentage close to the error percentage generally accepted after amplification, cloning, or sequencing. The general topology of the tree appears coherent with the classification issued from morphological data (*i. e. Idiosepius, Sepia,* and *Sepietta* grouped together) but none of the groupings is strongly supported by a high bootstrap value. *Idiosepius* is not more closely related to the Sepioidea than to the Teuthoidea. It must be stressed that a complementary analysis with all available species did not provide a clearer answer, the substitutions between families being saturated (Bonnaud *et al.*, 1994): *Idiosepius'* position cannot be determined.

Analysis of partial COIII gene

Analyses were performed with 166 informative sites out of 258 variable sites of a portion of cytochrome oxidase III gene. The trees obtained with nucleotide sequence analysis (Figs. 2-3) likewise show a solid grouping of the three loliginids. The sequences of the two populations of *Loligo vulgaris* differ significantly with this gene (4.9% of nucleotide divergence), and the divergence between *L. vulgaris* "Roscoff" and *L. reynaudii* was 6.3%. Their grouping was always supported by a very high bootstrap value (100).

Another well-supported group is composed of the oegopsid squid *Enoploteuthis reticulata* and of *Idiosepius pygmaeus*, the representative of Idiosepiidae, one of the sepioid families. This same grouping of *Idiosepius* and *Enoploteuthis* was obtained when taking all the substitutions into account or only the transversions. Grouping of the three other families of Sepioidea (Sepiidae, Spirulidae, and Sepiolidae) is not supported. This was confirmed by the separation of these three species in the PAUP analyses including all the substitutions or with weighted transversions (data not shown). When taking into account only the transversions, *Spirula* became linked with *Loligo* with a bootstrap value of 57.9 with Neighbor-Joining as well as with PAUP analyses, and the group excluding *Sepietta* and *Sepia* was well supported.

DISCUSSION AND CONCLUSION

It is clear that the two *Loligo* species are difficult to distinguish in terms of nucleotide variability with the

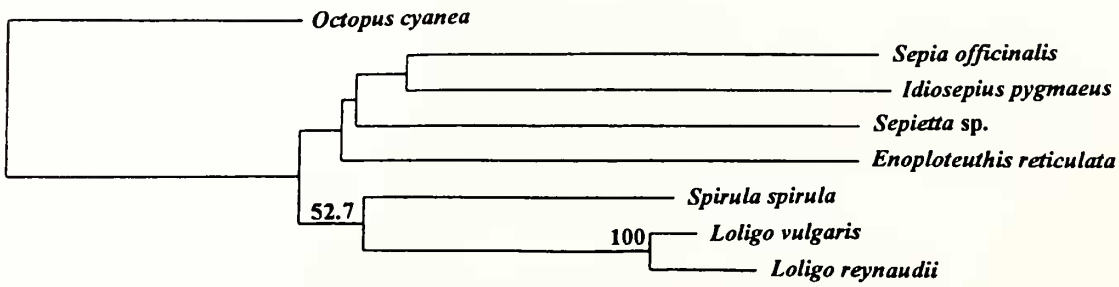
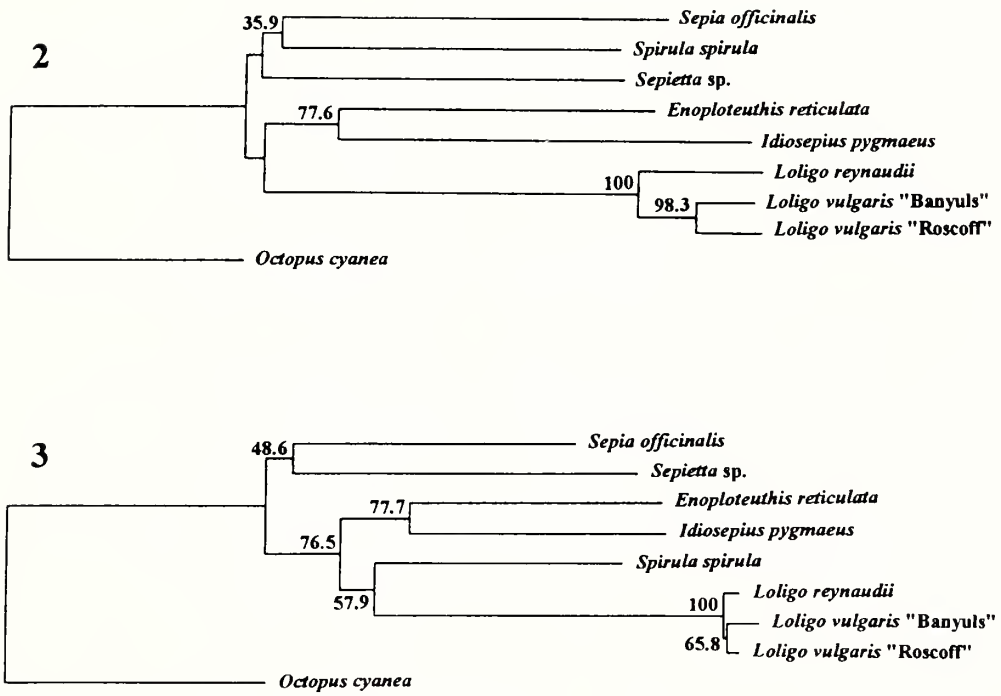


Fig. 1. Phylogenetic tree from the analysis of the 3' end of *l-r*-RNA using all substitutions (Neighbor-Joining method).



Figs. 2-3. Phylogenetic trees from analyses of partial COIII gene using (2) all the substitutions, (3) only the transversions (Neighbor-Joining method).

portions of the two mitochondrial genes. The observed divergence suggests differences due to geographical separation rather than a well-established speciation event. A study involving numerous specimens from along the French and African coasts would certainly provide an answer to this hypothesis of clinal variation.

The analysis of transversions only must be viewed with precaution, especially when the group excluding *Sepietta* and *Sepia* is considered. It is clear that the number of species was limited. In PAUP analyses of the COIII portion, *Sepietta* was linked with *Idiosepius* and *Enoploteuthis* whatever substitutions were considered. The opposing results from the two methods for the grouping of *Sepietta* reveal that this relation is uncertain and needs confirmation. The same is true for *Spirula* grouped with *Loligo*. The bootstrap values of 52.7 (with 16S) and 57.9 (with COIII) are low. If these values are really significant, they could be modified by increasing the species sampling, when possible.

On the contrary, *Idiosepius pygmaeus* is always grouped with the oegopsid irregardless of the analysis (parsimony or distance). This was unexpected because Idiosepiidae was placed by Naef (1916) with the Sepiidae and Sepiolidae as members of the order Sepioidea. For most authors, *Idiosepius* is more closely related to the Sepiolidae and Sepiadariidae than to the Sepiidae or Spirulidae, and its phylogenetic position has been questioned so far only with regard to the first two families (Fig. 4). The taxonomic rank of the idiosepiids has rarely been changed: it was always considered as a family of the order Sepioidea except by Guerra (1992) who raised Idiosepiidae to ordinal rank. The idiosepiids are isolated by characters like a dorsal adhesive organ in adults, the retardation of the tentacle development in juveniles, and the statocyst structure. They were often described without shell or gladius. The presence of a gladius was certainly difficult to detect because of the very small size of the members of this genus (20 mm maximum mantle length). Hylleberg and Nateewathana (1991a, b) found a thin gladius in the specimens examined and suggested that *I. pygmaeus* might be more closely related to Teuthoidea than to Sepioidea. Steenstrup (1881) created the genus *Idiosepius*; in his original description he mentioned that some specimens of small squids were described by early authors (*e. g.* Lamarck, Orbigny, Férussac, Blainville, Péron, Lesueur) under various names: *Cranchia minima* Férussac, 1835, *Loligo minima* Orbigny, 1848, *Loligopsis peronii* Lamarck, 1822, *Loligo parvula* Péron in Blainville, 1823, *Sepiola minima* Lesueur, 1821. In the opinion of Steenstrup, these decapods might be idiosepiids. The reasons which lead these early authors to attribute squid characteristics to *Idiosepius* could be an indication of the peculiar position of this genus within Decapoda. It is difficult to find morphological criteria which justify linking *Idiosepius* with sepiolids or sepiids and the few existing morphological studies, like those of Hylleberg and Nateewathana (1991a, b), do not analyze the taxonomic status of *Idiosepius*.

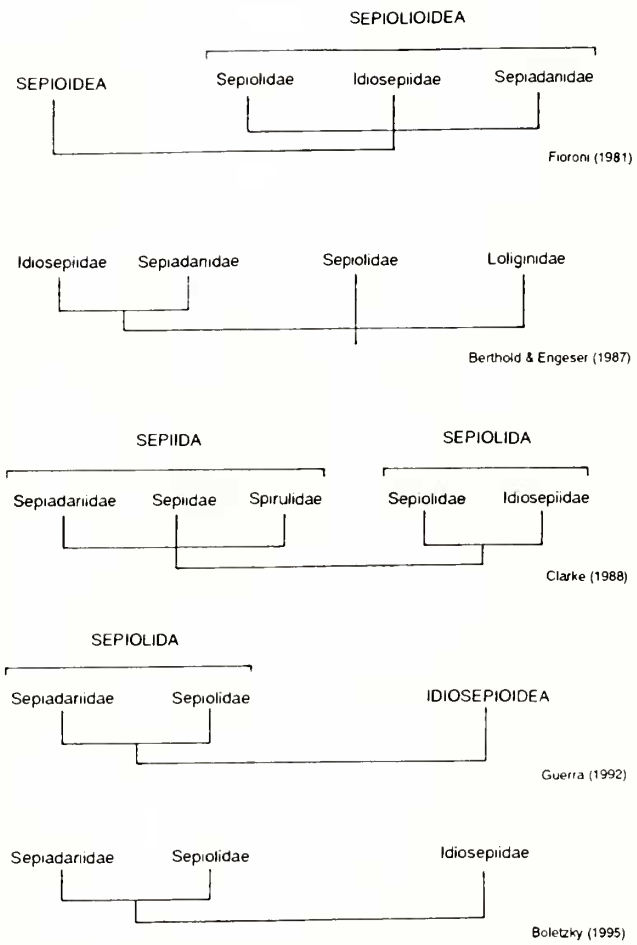


Fig. 4. Relationships of idiosepiids with other taxa according to various authors. Ordinal rank indicated by capital letters.

Analysis of additional species, and especially of other oegopsid families, might help to confirm the unexpected position of *I. pygmaeus*, and eventually to relate it more closely to an oegopsid family (Bonnaud *et al.*, in press).

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